Mineralization of erythromycin A in aquaculture sediments

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Abstract

Mineralization of erythromycin A was studied using two differently 14C-labeled erythromycins A, which were added to aquaculture sediment samples obtained from the two salmon hatchery sites in Washington state. The added erythromycin A did not significantly alter the numbers of the total viable colonies and erythromycin-resistant bacteria. Erythromycin-resistant Pseudomonas species contained a constitutive erythromycin esterase activity contributing to the inactivation of biologically active erythromycin A in aquatic and sediment environments. The initial rate of mineralization of erythromycin A appeared to be governed by the rate of release of soil-sorbed erythromycin A. After a prolonged lag time, the S-curves of erythromycin A mineralization were observed probably because of the increase in the population density metabolizing it. This study suggests that erythromycin A is partially or completely mineralized by the sediment microbial populations.

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1. Introduction

The extensive use of antimicrobial agents in food-producing animals has led to concern over the potential adverse effects of the antimicrobial residues on humans [1]. Among 21 veterinary and human antibiotics, erythromycin A is the most frequently detected one from 139 US stream sites considered susceptible to contamination from human, industrial, and wastewater [2]. Erythromycin A and its semisynthetic derivatives are the third most widely used class of antibiotics in animals and humans, and the great frequency of detection of erythromycin A may be due to the significant use in food-producing animals. Because erythromycin A is efficacious against a broad range of bacterial infections [3,4], erythromycin A is used to promote the growth of food-producing animals, and prevent bacterial diseases.

In fact, erythromycin is used in animal feeds for cattle, swine and poultry, but not for fish [5].

A large amount of antibiotics used in animal feeds may be delivered through wastewater and animal feces to sediments. The discharged erythromycin A is partly transformed to the dehydrated forms by acid- and base-catalyzed reactions in aquatic environments [6–8]. Erythromycin A is easily adsorbed on soil components, especially expandable clay minerals [9], and the sorption processes on soil components lend erythromycin compounds to persist in sediments [10]. The rate of degradation of soil-sorbed erythromycin A is greatly enhanced by the acidity of clay surfaces and the catalysis of clay minerals [11]. The behaviors of erythromycin A in sediment environments are important because the sorption and the chemical decomposition lead to inactivation of the biologically active compound.

The use of erythromycin A in animal feeds is a public health concern because of the possibilities of development of antibiotic-resistant bacteria and transfer of the resistance genes to human pathogens. However, little is known about the biological degradation and inactivation of erythromycin A by microbial populations in
Fig. 1. Structure of 14C-labeled erythromycin A. The 14C-carbons are asterisked at the N-methyl carbon position and the 1,3,5,7,9,11,13-carbon positions of the macrocyclic lactone.

2. Materials and methods

2.1. Chemicals

Erythromycin A was purchased from Aldrich Chemical Co., Milwaukee, WI. [1,3,5,7,9,11,13-14C]erythromycin A (specific activity, 0.327 mCi mmol⁻¹) was synthesized with purity >97% [12]. N-[methyl-14C]erythromycin A (specific activity, 54 mCi mmol⁻¹) with a purity of 98% was purchased from American Radiolabeled Chemicals, St. Louis, MO. Stock solutions were prepared by dilution with unlabeled erythromycin A in ethanol (2 μCi, 5 mg ml⁻¹), and preserved at −20 °C until used. Erythromycin A was added to sediment slurries after ethanol had been evaporated under gentle flow of sterile air. Oleandomycin (phosphate salt) was purchased from ICN Pharmaceuticals, Inc. (Costa Mesa, CA), and erythromycin A enol ether was prepared by the method of Kurath et al. [13].

2.2. Sediment samples

Sediment samples were collected by the Department of Fish and Wildlife in Washington State. Goldendale and Hupp Springs salmon hatcheries, denoted as GD and HS, were chosen to represent an erythromycin treated site and a non-treated control site, respectively. GD hatchery is located on the Klickitat River on its way south to the Columbia River, and HS hatchery is located on the White River on its way to the Puget Sound. GD hatchery had not used medicated feed for at least 6 years before sampling in October, 1998. HS hatchery was treated with erythromycin-medicated feeds for 3 years until June, 1998. The wet sediment slurries were sieved to remove the >2 mm particles, and stored at 4 °C until used. The sediment samples were dried at 60 ± 5 °C, and placed in a desiccator until the weight became constant. The particle size distributions were determined according to the guide of the US Department of Agriculture (USDA) using a soil hydrometer (Fisher Scientific, Pittsburgh, PA). The percent contents of organic matters were determined after 4 h of the ignition of dried soils at 450 °C.

2.3. Microbial population analysis

At the beginning and after 230 days of incubation, 100 μl slurry samples were taken from sediment microcosm tanks immediately after vigorous shaking, and the 10⁻¹⁻¹⁰-fold serial dilutions were prepared with sterile 0.85% NaCl [14]. Aliquots (100 μl) of the serial dilutions were evenly plated on tryptic soy agar plates (Remel, Lenexa, KS) in the presence or absence of erythromycin A (final conc., 25 μg ml⁻¹). The plates were incubated for 3 weeks at 10 °C, and plates containing 30–300 colonies were used in determining the colony forming units (CFU) per gram-sediment. Among them, characteristic colony forms (e.g., colony size, color and shape) were selected, and isolations of the single colonies were repeated using the same agar medium. The minimum inhibitory concentrations (MICs) of erythromycin A to selected erythromycin-resistant (Emr) strains were determined using 96-well microplates containing tryptic soy broth as a basal medium. Concentrations of erythromycin A in the 11 wells were serially 2-fold diluted from 1600 to 0.78 μg ml⁻¹, and no erythromycin A was added in a reference well. Microplates were homogeneously inoculated with the initial optical density of 0.05–0.1 at 600 nm, and were incubated for 24 h at 30 °C. The MIC₉₀ was determined from 90% difference of the optical density (ΔOD₆₀₀) of a treated well against the reference well. Respiratory finger-prints of highly Emr strains with the MIC₉₀ of more than 1600 μg ml⁻¹ were examined using GN MicroPlates™ (Biolog, Hayward, CA), and the identification was performed using GN Data Base after 4 h of the incubation at 30 °C.

2.4. Erythromycin esterase assay and analytical conditions

Highly erythromycin-resistant bacterial isolates were cultivated for 18 h in 100 ml tryptic soy broth on a rotary shaker (180 rpm) at 25 °C. Cells were harvested by centrifugation at 4 °C and 14,000g for 15 min, and washed three times with 50 mM sodium phosphate buffer (pH 7.4). Cells were disrupted by five cycles of 30-s sonication and 30-s cooling on ice. The cell debris was removed by centrifugation at 4 °C and 120,000g for 1 h, and the supernatants were used for the assay of eryth-
romycin esterase activities to erythromycin A, erythromycin A enol ether and oleandomycin. The enzyme reactions were determined by reversed phase high performance liquid chromatography with electrochemical detection, as previously described by Kim et al. [15].

The enzymatic reaction was confirmed from the analyses of erythromycin A and its degradation product by the LC/ESI-mass spectrometry and the $^{13}$C NMR spectrometry. The mass analyses were performed on an HP 5989B mass spectrometer equipped with an HP 1090L/M HPLC (Hewlett-Packard, Palo Alto, CA). The mass spectrometer was operated in electrospray mode with the capillary exit voltage of +100 V for positive ions or −100 V for negative ions. Full scans were acquired from m/z 50 to 900 at 0.92 scans/s for all analyses. Compounds were resolved using a Prodigy ODS3 100A HPLC column, 5 μm, 2.0 × 250 mm, (Phenomenex, Torrance, CA). The mobile phase, deuterated water (H2O)/C176 methanol (MeOH) 50:50 to 90:10 at 100 μl/min was used for the analyses. The mobile phase (H2O)/C176 methanol (MeOH) 50:50 to 90:10 at 100 μl/min was used for the analyses. The mobile phase was composed of 100 mM NaCl, 0.1 M KH2PO4, 0.1 M KH2PO4 at pH 7.0, and 0.1 M KH2PO4 at pH 7.0, with constant 3 mM ammonium formate. The $^{13}$C NMR spectra were recorded on a 300.075 MHz Varian NMR spectrometer (Palo Alto, CA) in CDCl3 with employing proton decoupling during data acquisition. Parameters were as follows: spectral width, 20,000 Hz; 90° pulse, 13 μs; relaxation delay, 1 s; relaxation time, 1.49 s.

2.5. Sorption and desorption experiments

To obtain sorption isotherms using $N$-[methyl-$^{14}$C]erythromycin A, one gram (dry wt.) of sediment slurry was suspended in 20 ml of 0.01 M CaCl2 solution with various concentrations of 3.41−34.1 μM erythromycin A (0.1−1.0 μCi) in 50 ml teflon centrifuge tubes. Control tubes without sediment slurries were prepared in the same manner to calculate the amounts sorbed on vessels. After shaking for 3 days at 10 °C and 30 rpm, suspensions were centrifuged at 10 °C and 14,000g for 15 min. The amount of soil-sorbed erythromycin A ($C_s$ μmol kg$^{-1}$) was calculated from the difference of the radioactivity (dpm) in supernatants against the initial concentrations ($C_i$). Sorption isotherms were fit to the logarithmic Freundlich equation: $\log C_s = \log K_f + n_f \log C_i$, where $C_e$ (μmol l$^{-1}$) was the equilibrium concentration of erythromycin A, $K_f$ and $n_f$ were the Freundlich sorption coefficients. When erythromycin A was strongly adsorbed ($C_s \gg C_e$), the equilibrium concentration of erythromycin A was much smaller than the initial concentration. Thus, the Freundlich equation simply approximated the equation, $C_i = K_f \cdot C_i^{n_f}$, to calculate the concentration of soluble erythromycin A at a given concentration. Rates of desorption of soil-sorbed erythromycin A (μmol g$^{-1}$ d$^{-1}$) were determined at 10 °C from aqueous soils containing 1 g soil and 20 ml of 0.01 M CaCl2, to which 34.1 μCi erythromycin A (1.0 μCi) was added. After the equilibration, soil particles were centrifuged at 10 °C and 14,000g for 15 min, and the supernatants were replaced with equal volumes of 0.01 M CaCl2. The desorption–centrifugation procedure was repeated until the constant rate of desorption from the inner lattices of expandable clays was observed [16].

2.6. Mineralization experiments

An experimental condition can greatly impact the mineralization studies apart from the reality, particularly when the examined inoculum is a mixed culture [17]. Therefore, 30 ml pond water was used in suspending 10-g (dry wt.) of sediment in 250 ml-biometer flasks, in which 160 μl of $N$-[methyl-$^{14}$C]erythromycin A stock solution (final conc. 20 μg ml$^{-1}$) was added. In the side arm, 10 ml of 0.1 M KOH was filled to trap CO2 evolved from the main flask compartment. Abiotic slurries were prepared by autoclaving for 15 min at 121 °C before spiking erythromycin A, and were treated with sodium azide (final conc., 0.002%) to prevent the contamination during the experiment period. The test and control flasks were prepared in triplicate, and were placed on a rotary shaker (30 rpm) for 15 days at 10 °C. To measure the $^{14}$CO2 evolved from slurries, 1 ml of trapping solution was replaced with the same amount of 0.1 M KOH solution at 0, 1, 2, 3, 4, 8, 11, and 15 days. Alternatively, the batch mineralization experiments were performed using an air flowthrough microcosm test system, which was designed to collect the evolved CO2 without disturbing sediments [14]. Ten grams (dry wt.) of sediment and 200 ml of pond water were mixed in a 0.5-liter microcosm tank, in which either 800 μl of [1,3,5,7,9,11,13-$^{14}$C]erythromycin A stock solution (final conc. 20 μg ml$^{-1}$) or 2 ml of $N$-[methyl-$^{14}$C]erythromycin A stock solution (final conc. 50 μg ml$^{-1}$) was added. Control microcosm tanks were prepared as described above in biometer flask experiments. Test and control microcosm tanks were prepared in duplicate, and were placed in a dark room at 10 °C. The spatial air was passed through a volatile organic trapping column containing 2 cm of polyurethane foam and 500 mg of Tenax TA polymer (20/35 mesh, Alltech Associates, Inc., Deerfield, IL) with a constant rate of ca. 10 ml min$^{-1}$, and the CO2 was trapped in 50 ml of 70% monoethanolamine −30% ethylene glycol [14].

2.7. Measurements of radioactivity

One milliliter CO2-trapping solution was thoroughly mixed with 15 ml scintillation cocktail (Ultimagold, Packard Instrument Co., Meriden, CT) to measure the radioactivity (dpm) with a Packard 2000CA Tri-Carb liquid scintillation counter (Packard Instrument Co., Downers Grove, IL). The initial background was measured with a blank vial containing 1 ml unexposed trapping solution and 15 ml scintillation cocktail.
2.8. Statistical analysis

All data points were reported as the mean values and the standard errors of the means (SEM). Statistically significant differences among the treatments were determined using the analysis of variance (ANOVA) procedure and the Tukey’s w-procedure with 95% confidence limits.

3. Results

3.1. Physicalchemical properties of sediments

The physical and chemical properties of GD and HS sediment samples were similar. GD and HS consisted of sand, silt and clay in ratios of 93.4:4.8:1.8, and 90.7:7.8:1.5, respectively. GD contained more organic matter (2.9%) than HS (1.6%). The pH values of the bulk solutions in GD and HS were measured at 6.6 and 6.7, respectively. According to the classification of the sorption isotherms [18], the sorption isotherms of erythromycin A on GD and HS sediments were of the L-type, because the values of $K_L$ were estimated at 52 ± 0.02 and 0.43 ± 0.016, respectively. The sorption capacities, $K_L$ were calculated at 815 ± 48 for GD and 481 ± 23 for HS. The clay and organic matter contents appeared to be positively related to the sediment capacity of adsorption of erythromycin A.

Rates of desorption of soil-sorbed erythromycin A became constant after three replacements of the supernatants with equal volumes of 0.01 M CaCl$_2$ (Fig. 2). A large amount of erythromycin A added to sediment sample seemed to be strongly adsorbed in the inner lattices of expandable clays, as observed with $n$-alkylamines bound on clays [16]. The linear desorption rates of soil-sorbed erythromycin A from GD and HS sediment samples were estimated at 1.63 ± 0.10 and 3.48 ± 0.65 nmol g$^{-1}$ day$^{-1}$, respectively. The rates of release of soil-sorbed erythromycin A appeared to be inversely related to the contents of clay and organic matter in the sediments.

3.2. Analysis of total viable bacteria and erythromycin-resistant bacteria

The numbers of total viable colonies (CFU per g-soil) and erythromycin-resistant (Em$^r$) bacteria in GD and HS microcosm samples at the beginning and after 230 days of the incubation in the presence of 20 or 50 µg ml$^{-1}$ erythromycin A are given in Table 1. The two sampling times at zero time and 230 days showed no significant difference in the colony-forming units per g-soil ($p > 0.05$), which were accounted for (5.3 ± 2.1) x 10$^6$ and (4.5 ± 1.6) x 10$^6$ in GD and HS samples, respectively. From tryptic-soy agar culture plates, 19 types of the colonies were isolated from the GD samples, and 15 types of the colonies were isolated from the HS samples. Among them, 14 GD isolates (74%) and 13 HS isolates (87%) were resistant to erythromycin A. The given erythromycin A concentrations at 20 and 50 µg ml$^{-1}$ did not significantly change the numbers of Em$^r$ bacteria either ($p > 0.05$), which were accounted for (1.6 ± 0.25) x 10$^6$ and (3.4 ± 0.89) x 10$^6$ in GD and HS samples, respectively. No significant change in the size of sediment microbial populations and Em$^r$ bacteria may be due to a low level of erythromycin A which was not adsorbed on soil components. The soluble erythromycin A concentrations ranged from 1.1 to 6.2 ng ml$^{-1}$ in the GD samples and from 0.92 to 7.8 ng ml$^{-1}$ in the HS samples.

Among the 27 Em$^r$ strains, 11 strains were highly resistant to erythromycin A (MIC$\geq$1600 µg ml$^{-1}$). All of these strains were Gram-negative motile rods, and grew optimally at 15-25 °C. From the duplicate tests using GN MicroPlates™ and Data Base, 10 strains had similarities with the members of the genus Pseudomonas, but one strain was not identified. The cell-free extracts of these strains contained a constitutive erythromycin esterase activity, which was involved in the degradation of erythromycin A, erythromycin A enol ether and oleandomycin [15].

When the erythromycin A degradation product was separated by a reversed phase HPLC (Fig. 3), the ESI-mass spectra of erythromycin A (I) and the degradation product (II) showed the positive molecular ions [M + H]$^+$ of m/z 734 and m/z 752, respectively, with the capillary exit voltage of +100 V, and the negative molecular ions [M − H]$^-$ were detected at m/z 732 and m/z 750 with the capillary exit voltage of −100 V. Accordingly, the respective molecular weights of erythromycin A (I) and the degradation product (II) were 733 and 751, respectively. The increase of 18 indicated the addition of water on the erythromycin A structure. By the $^{13}$C NMR analyses, erythromycin A (I) in CDCl$_3$ showed chemical
shifts (ppm) at 217.16, 170.92, 78.67, 74.99, 73.00, 72.54, 72.11, 70.00, 69.70, 67.71, 65.96, 63.97, 60.71, 60.60, 44.57, 40.25, 39.97, 35.38, 34.48, 33.55, 32.88, 30.00, 23.94, 21.98, 16.57, 16.45, 16.16, 13.68, 13.35, 11.25, 11.04, 7.11, 5.74 and 4.24. The $^{13}$C NMR spectrum of the degradation product (II) in CDCl$_3$ showed chemical shifts (ppm) at 195, 103.14, 94.08, 71.5, 71.3, 69.37, 69.08, 67.79, 65.14, 64.41, 60.56, 59.58, 44.53, 35.13, 34.57, 33.36, 30.35, 29.37, 17.93, 17.05, 16.83, 16.71, 16.07, 14.14, 13.12, 12.84, 8.56, 7.23, 6.44, 6.13, 5.35 and 4.60. The one-dimensional carbon peaks of the product (II) were consistent with the structure of erythromycin A-1-carboxylic acid. These spectral data proved that the erythromycin esterase catalyzed the hydrolysis of the macrocyclic lactones of erythromycin A.

### 3.3. Biometer flask experiments

The mineralization of $N$-[methyl-$^{14}$C]erythromycin A showed the biphasicity from GD and HS biometer flasks (Fig. 4). The HS sediment appeared to be more potent for mineralizing $N$-[methyl-$^{14}$C]erythromycin A than the GD sediment. The biphasicity might be related to the sorption processes of erythromycin A in sediments, because the rate of release of soil-sorbed erythromycin A from the HS sediment was greater than that from GD sediment.

### 3.4. Microcosm experiments

The mineralization curves for erythromycin A in GD and HS microcosms are given in Figs. 5 and 6, respectively. The $^{14}$CO$_2$ production from the abiotic control tanks was minimal. The mineralization in both sediments treated with either of two differently

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**Table 1**

<table>
<thead>
<tr>
<th>Sediment samples</th>
<th>Sampling time (days) + added EM conc.</th>
<th>Colony-forming units ($10^5$ CFU per g-soil)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>+No. EM</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+25 μg ml$^{-1}$ EM</td>
</tr>
<tr>
<td>GD</td>
<td>0 day</td>
<td>53(39)</td>
</tr>
<tr>
<td></td>
<td>230 days + 20 μg ml$^{-1}$ EM</td>
<td>30(23)</td>
</tr>
<tr>
<td></td>
<td>230 days + 50 μg ml$^{-1}$ EM</td>
<td>75(60)</td>
</tr>
<tr>
<td>HS</td>
<td>0 day</td>
<td>46(37)</td>
</tr>
<tr>
<td></td>
<td>230 days + 20 μg ml$^{-1}$ EM</td>
<td>54(41)</td>
</tr>
<tr>
<td></td>
<td>230 days + 50 μg ml$^{-1}$ EM</td>
<td>34(29)</td>
</tr>
</tbody>
</table>

The standard errors of means are given in parentheses.

$^a$CFU, colony-forming units (CFU) determined on tryptic soy agar plates in the presence or absence of 25 μg ml$^{-1}$ erythromycin A (EM).
14C-labeled erythromycins A displayed a prolonged lag time of about 120 days. During the lag periods, the biphasic curves were observed (Figs. 5 and 6 insets), as seen in the biometer flask experiments.

After the lag periods, N-[methyl-14C]erythromycin A was mineralized near to 100% from GD microcosm tanks, but [1,3,5,7,9,11,13-14C]erythromycin A was not actively mineralized. In contrast, 90% of [N-methyl-14C]erythromycin A and 75% of [1,3,5,7,9,11,13-14C]erythromycin A were mineralized from HS microcosm tanks. From the analysis of variance (ANOVA), the mineralization curves for the two differently 14C-labeled erythromycins A in the HS microcosm tanks were significantly different from one another (p < 0.05). The results indicated that different microbial populations were involved in the partial degradation of the N-methyl group and the macrocyclic lactone of erythromycin A. From the microcosm experiments, the HS sediment had more potential activities for mineralizing erythromycin A than the GD sediment. This may be due to the fact that the HS sediment was previously exposed to erythromycin A in the hatchery site.

4. Discussion

The use of antibiotics in food-producing animals is an emerging issue for the food safety and public health, because of the possible developments of antibiotic resistant bacteria and transfer of the resistance genes to human pathogens [19]. Large amounts of antibiotics used in food-producing animals enter soils and sediments through the discharge from animal guts and farm wastes. Not only the intestinal microflora of animals treated with antibiotics, but also soils and sediments contaminated from the effluents can serve as reservoirs of resistant microorganisms [1].

In this study, GD and HS sediment samples spiked with erythromycin A (20–50 µg ml⁻¹) did not show any significant change in the numbers of total viable colonies and erythromycin-resistant bacteria at zero and 230-day sampling times. From both sediment samples, the predominance of erythromycin-resistant bacteria were found. Of them, Gram-negative bacteria, which are capable of hydrolyzing macrolide antibiotics by their constitutive erythromycin esterases, appear to play a role in lowering the level of biologically active erythromycin A in aquatic and sediment environments [15,20,21].

In aquatic systems, the solubility of chemicals and the presence of sorptive materials, such as clays and colloidal organic matter, affect the biodegradation [22]. Since the protonated species of erythromycin A (pKₐ = 8.36) can be strongly adsorbed to clay particles [9,11], the soil-sorbed molecules are less likely accessible to microorganisms. Thus, biphasicity of the mineralization of erythromycin A indicates that the biodegradation rate is governed by the desorption rate of soil-sorbed erythromycin A. The biphasic mineralization curves are usually observed from the degradation of poorly soluble hydrocarbons whose the degradation rates are limited by the dissolution rates [23] and the mass transfer rates [24,25].

From microcosm experiments, the observed S-curves for the mineralization of erythromycin A seem to be related to the increase in the density of microbial populations metabolizing it. Generally, a prolonged lag time can be caused by the disruption of the sediment profiles and the dynamic changes in the composition of organic matter and microbial populations. In addition, Schmidt et al. [26] suggested that the growth on other substrates in sediments displayed sigmoidal mineralization curves, if low chemical concentrations could not support the growth of actively metabolizing populations.
Using the two differently $^{14}$C-labeled erythromycins A, this study demonstrates that the macrocyclic lactone and the N-methyl group are partially or completely degraded by the sediment microbial populations, and that the macrocyclic lactone is rather persistent than the N-methyl group. From the two different sediments tested in this study, HS sediment with the previous exposure to erythromycin A included the microbial populations metabolizing it completely, whereas GD sediment microbial populations which had no previous exposure to antibiotics had only partial activity for degradation of the N-methyl group. In both cases, the sediment microbial populations can inactivate biologically active erythromycin A by either partial or complete degradation.

In conclusion, erythromycin A can be biologically and chemically degraded (inactivated) and mineralized in sediments whether or not they have been exposed to this class antibiotics. Further studies will be necessary to determine the degradation of erythromycin used in animal feeds and aquaculture with different loadings.

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